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(54) Title: IMPROVED METHOD OF IMMUNIZATION

(57) Abstract: A method for improving the immune response to a immunogen by separately administering to a patient an immunogen composition for sustained release comprising an epitope of the immunogen target, and a supplement comprising an adjuvant compound for stimulating, potentiating or activating a strong immune response. The provided method potentially reduces local irritation at the sites of inoculation, i.m. or s.c.

IMPROVED METHOD OF IMMUNIZATION

The present application claims priority from the Provisional U.S. Application 60/164,054 filed November 8, 1999.

FIELD OF INVENTION

The invention relates to an improved method of immunization, and in particular, to a method of preparing an immunizing composition for separate injection of an immunogenic portion of and an adjuvant portion of the composition.

BACKGROUND OF INVENTION

Antigenic compositions or vaccines are formulated to be effective in immunizing an animal or a human against invading foreign organisms or matter or infective agents by eliciting an immune response which culminates, inter alia, in the production of antibodies directed against the invading organisms or matter or infective agents. Thus, the compositions elicit antibodies that are specifically directed against the antigenic aspects of the foreign substances or microorganism or other infective agents, including pathogenic organisms, toxic products of said organisms, and viruses.

The success of an antigenic composition is linked to its immunogenicity, that is, the ability to produce a sufficiently high or effective titer of antibodies to bind to the target pathogen or pathogenic toxins. Thus, the immunogenicity depends on the effectiveness by which the antigen causes the body's immune system to elicit a response. The immune response is generally assessed on the basis of the antibody titer in the blood of the immunized animal or mammal including the human. Immunizing formulations targeted against antigens of low immunogenicity are usually constructs or mixtures of the select immunomimic epitope of a target antigen and an immunogenic carrier component not related to the target antigen. The immunogenic component aids or causes a strong immune response against the entire construct or mixture so as to be effective against the target antigen. Supplemental components, in the context of the invention, are adjuvants, such as, e.g., bacterial extracts or specific synthesized products.

Thus, the antibodies elicited by the antigenic formulation can bind to specific targets on the cell surfaces or free circulatory proteins. Specific antibody populations can act as biological modifying or interfering agents affecting the physiological activities of the body, the cells tissues or by binding to receptors, enzymes, signal transducing peptides or other proteins. Cell cycle control or checkpoints and numerous biological activities or events can be affected by the specifically targeted antibodies.

In order to further enhance or potentiate the immune defense system, so-called adjuvants in

the form of oily substances and other potentiating agents are conventionally added to the antigenic formulations. Adjuvants are usually mixed into the immunogenic formulation and simultaneously delivered with the antigen in the same administration, e.g., by injection. In particular, the formulations have been enhanced to raise effective antibody titers against fractions of microorganisms or viral pathogens by the addition of so-called adjuvants. Adjuvants are compositions comprising immune response-stimulating killed microbial cells, particles, fragments or components thereof. Moreover, immunogenic compositions can contain different kinds of carrier, including emulsions, liposomes, microparticles and implantable vehicles which are, moreover, metabolizable.

More recently developed anti-infective agent immunization technology as described above, has been applied as a modifying biological means to immunize against various soluble and insoluble animal, including human, antigens normally not recognized or targeted by the individual's own immune defense, but which can be rendered immunogenic so as to significantly stimulate or potentiate the individual's own immune response system against itself. The self-antigens can include the surfaces of certain cells which are dysfunctional or malignant, and small proteins, enzymes or intercellular signals, such as, e.g., hormones or other signal factors. The lack of effective immunogenicity of these self-antigens can often be overcome by complexing or linking the non-immunogenic self-antigens with a pharmaceutically acceptable, i.e. non-toxic, immunogenic carrier so as to produce antibodies capable of binding, thereby neutralizing, the self-antigen epitope of the subject animal or human patient.

Immunization against hormones in mammalian and, in particular, human subjects, has been therapeutically applied to control or modify hormonal effects on the physiology of the body. In many cases, as disclosed e.g., in U.S. Patent Nos. 5,688,506, 4,526,716, 4,676,981, 5,023,077, 5,891,792, 5,843,446, and 5,723,129, the immunization procedure requires repeated injections of formulations containing an immunogen and an immune response stimulating adjuvant.

The immunomimic portion of the immunogen construct can encompass epitopes of both a non-peptide and peptide variety so that the immune reaction is directed to the structural and/or functional properties of the target hormones or other factors. The immunological methods for the therapeutical hormone action control or modification can be used for the treatment of patients afflicted with a disorder or disease. For example, the immunological method for the control of a peptide hormone can control certain physiological activity in normal or disease-afflicted subjects.

Some immunogenic constructs suitable for hormone-controlling or modifying effect comprise immunomimicking molecular moieties of the hormones which are conjugated or fused to immunogenic carriers, which may comprise complex polysugars, proteins or peptides. The

immunogenic constructs are usually administered in the form of an emulsion, either an oil-in-water or a water-in-oil emulsion, containing an adjuvant capable of stimulating or potentiating an immune response. A conventional methodology includes mixing the preparation of an antigenic emulsion compositions. For example, a water-in-oil emulsion is usually prepared by mixing equal parts or ratios of 70:30 of an aqueous solution of an antigen with Freund's Complete Adjuvant containing inactivated mycobacteria in a suitable oily substance (i.e., mineral oil). Since the Freund's Complete Adjuvant is very irritating in the human subject, pharmaceutically more amenable oily substances other than mineral oil have come into use. Acceptable oil phase vehicles have been selected from metabolizable oily substances.

An immune response is typically measured in terms of the production of specific antibodies. For example, the hormones which are targeted for control by the immunological methods are directly neutralized by the antigen-binding reaction with circulating specific antibodies elicited by the immunogenic constructs. Other affected hormones, forming cascades of hormonal activities upon the signal of the primary hormone.

An anti-hormone immunogen has been constructed to affect the regulation of the gonadotropin releasing hormone (co-assigned U.S. Patent 5,688,506). The Gonadotropin Releasing Hormone (abbreviated "GnRH", also known as Luteinizing Hormone Releasing Hormone, abbreviated "LHRH"), is of central importance to the regulation of fertility. Johnson M et al., *Essential Reproduction*, 3rd Edn. Blackwell Scientific Publications (1988). In both males and females, GnRH is released from the hypothalamus into the bloodstream and is transported through the bloodstream to the pituitary, where it induces the release of gonadotropins, luteinizing hormone and follicle stimulating hormone, by the gonadotrophs. These gonadotropins, in turn, act upon the gonads, inducing steroidogenesis and gametogenesis. Steroids released from the gonads into the circulation subsequently act upon various tissues. This gonadotropin related hormonal cascade can be halted by neutralization of the biological activity of GnRH. Fraser H.M., Physiological Effects of Antibody to Lutenizing Hormone Releasing Hormone, *Physiological Effects of Immunity Against Reproductive Hormones*, Edwards and Johnson, Eds. Cambridge University Press (1976). As a consequence of GnRH neutralization, the gonadotropins and gonadal steroids are not released into the blood, and their biological activities are curtailed or eliminated by the direct and indirect action of specific anti-GnRH antibodies. By eliminating the physiological activity of GnRH, the cascade of hormonal regulation of fertility is interrupted and gametogenesis ceases. Consequently, GnRH neutralization halts the production of gametes. Thus, GnRH neutralization is an effective means of contraception.

Furthermore, a number of important diseases are affected by gonadotropins and particularly

gonadal steroid hormones. Such diseases include breast cancer, uterine and other gynecological cancers, endometriosis, uterine fibroids, benign prostatic hypertrophy and prostate cancer, among others. Removal of the gonadal steroid hormonal stimuli for these diseases constitutes an important means of therapy. An effective method of accomplishing this is by immunologically neutralizing GnRH, to thereby eliminate gonadal steroids that induce and stimulate these diseases. McLachlan R.I. et al. Clinical Aspects of LHRH Analogues in Gynaecology: a Review, *British Journal of Obstetrics and Gynaecology*, 93:431-454 (1986); Conn P.M. et al. Gonadotropin-Releasing Hormone and Its Analogs, *New England Journal of Medicine*. 324:93-103 (1991) and Filicori M. GnRH Agonists and Antagonists, Current Clinical Status. *Drugs*. 35:63-82 (1988).

Since GnRH has the same amino acid sequence in all mammals (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2), it is presumed that a single immunogen would be effective in all mammalian species, including humans. The so-called active form of immunization has required a composition of immunogens against GnRH which elicits a sufficient titer of anti-GnRH antibodies in the patient to physiologically neutralize and thereby limit the hormone level in the patient.

Thus, the anti-GnRH directed conjugate of certain analogous peptides to the GnRH immunomimic domain can be linked to a carrier protein which is effectively immunogenic, such as, e.g., diphtheria toxoid, tetanus toxoid, keylimpet hemocyanin, bovine serum albumin, Hemophilae pertussis extracts or filamentous Amycolata extracts. Consequently, the immune response to GnRH-vaccine will be primarily directed against the carrier protein and secondarily, the attached hormone epitope moiety (hapten).

As previously discussed, the Freund's Complete Adjuvant is accepted as an unofficial standard of efficacy for inducing an immune response. However, it is not suitable for use in humans since it is very reactogenic and irritating. Amenable adjuvants, therefore, have been chosen from non-toxic tissue-amenable compounds or compositions, such as, e.g., norMDP or MDP. However, even with acceptable adjuvants, the inoculations often cause a local inflammatory reaction which can be very discomforting or painful to the patient. Tissue reactogenicity is a particularly undesirable side effect when the administration of the vaccine must be repeated in order to maintain or boost the antibody titer, often leading to more severe irritation of the injection sites potentially resulting in painful lesions. Moreover, in order to obtain the desired level of the immune response to an immunogenic construct, a relatively large amount of adjuvant is sometimes required, thereby often aggravating the local tissue reaction of both i.m. and s.c. injection site. Finally, the accompanying tissue reaction of a large inoculum can lead to limited immune response to the target antigen.

Therefore, it is an object of this invention to provide delivery of the adjuvant portion of an immunogenic composition separately from the immunogen, while providing adjuvant driven

enhancement of the immune response against the antigenic domains or components of the immunogenic composition. In particular, it is an object of this invention to increase the efficacy of immunization, by an improved procedure which has the potential to reduce local irritation and other side effects without loss of efficacy. It is a further object of the invention to provide separate preparations of immunogen and supplement for immunization against hormonal or non-hormonal antigens.

SUMMARY OF THE INVENTION

The present invention is directed to an improved method for the immunization of an animal or mammal including the human whereby the local tissue irritation of the injection site may be reduced while the immune response is advantageously enhanced.

The improved method has been surprisingly effective in enhancing a high antibody titer, such that the amount of immunomimicking portion of the immunogen can be reduced resulting in more gentle and economic immunization treatments as well as other advantages affecting the immune response.

According to the invention, the improved method comprises the separate administration of an immunogenic portion comprising the immunogen and a potentiating supplement portion comprising the immunostimulating additive or adjuvant.

Thus, with the invention, a single adjuvant formulation can be used to enhance the immune response to each of many different immunogens. Moreover, the dose of adjuvant can be selectively varied for a specific application of each immunogen which enables the use of a single immunogenic formulation with selected administration of a separate adjuvant. A further advantage of the present invention is found in separate administration steps to help overcome any incompatibility between adjuvant and immunogen formulation components.

Another embodiment according to the invention provides the selection of different adjuvants for each dosing of a immunizing composition. Separate administration of adjuvants also affords formulation of adjuvant compositions incorporating suitable characteristics for *in vivo* particle release rates different from the immunogenic components. Consequently, the adjuvant driven enhancement or amplification of the immune response can be optimized for each immunization step.

Another advantage of the present invention can be found in that adjuvants can be administered for selected administrations of immunogenic constructs in order to optimize the immunopotentiation of the antibody response.

In addition, the adjuvant formulations for the separate administration, as provided by this invention, comprise sustained release formulations, including emulsions, liposomes, microparticles and implantable vehicles, as are known in the pharmacological art.

The immune response can be further manipulated by varying the timing of adjuvant supplement administration with regard to the immunization protocol. In particular, the adjuvant compositions can be administered at suitable anatomical sites located near or distant from the immunization site itself.

Furthermore, immunostimulating adjuvants can be administered by different routes so as to optimize the immunopotentiating activity of the adjuvant for a given immunogen. In particular, the routes of administering an adjuvant to a patient in need thereof can be identical or different from the immunization. Thus, the separate injections of immunogenic composition and adjuvant composition can be located in close proximity adjacent to each other, in accordance with an embodiment of this invention.

According to invention, the separately administered immunizing portions comprise firstly a primary emulsion containing the immunogenic portion (also referred to as the immunogen) and secondly, a secondary emulsion containing an immunostimulating adjuvant (also referred to as the supplement).

The present invention provides separate administrations of biocompatible adjuvants which can be muramyl dipeptide (MDP) derivatives, including norMDP and threonyl MDP. Other suitable adjuvant preparations can utilize lipopolysaccharide (LPS) derivatives, including soluble lipopolysaccharide (SLPS) for use in separate adjuvant administrations. Dependent on the chemical or physical nature of the adjuvants, the formulations for separate administrations may include aqueous solutions, which can contain sterile water, saline or phosphate buffered saline. Preferably, the aqueous solution are neutral isotonic compositions.

In accordance with the present invention, a vaccination kit can be provided for increasing the immune response to a target vaccine wherein the kit comprises in separate aseptic preparations or entities, an immunogenic composition and an immune response stimulating or potentiating composition. The preparations can be provided under aseptic conditions.

Another embodiment according to the invention provides an improved method for effectively modifying, controlling, or inhibiting the physiological function of a mammalian or human hormone by separately administering an immunizing formulation comprising an anti-hormone immunogenic construct and another kind of vaccine formulation comprising an immune response stimulating additive or adjuvant. The separate administration of the supplement is provided so as to reduce or avoid side reactions such as severe tissue reaction or inflammation while enhancing the immune response to the immogen. The invention, therefore, provides that the primary part of the immunogenic water-in-oil emulsion contains an anti-hormone immunogenic conjugate and a secondary part of the water-in-oil vaccine emulsion contains a preferred norMDP adjuvant or

supplement. The immunogenic construct portion is administered at a site which is administered to the same tissue group or organ but is separate from the inoculation site of the supplement comprising the immunostimulating or immunopotentiating adjuvant emulsion. The novel method of separate injections is thus providing an immunization more effective for generating an immune response and potentially less stressful for the subject animal or patient.

In accordance with another embodiment of the invention, the initial immunogen or vaccine can be delivered by conventional methods, namely, by a single parenteral injection of an emulsion mixture of the immunogenic conjugate, which also contains an immunostimulating additive, such as, e.g., norMDP, and the oily substance acceptable for human use as, e.g., Montanide ISA 703, Montanide 25, Montanide ISA 719, or Montanide ISA 720 (Seppic). The initial immunization step is, however, followed by booster injections at two separate locations on the suitable tissue. The booster injections comprise an immunogenic emulsion prepared from a mixture of an aqueous solution of an immunomimic antigen, which is linked to an immunogenic carrier, and emulsified with a pharmaceutically acceptable oily substance such as e.g., Montanide ISA 703, Montanide ISA 719 or Montanide ISA 720. The second or supplement emulsion comprises a mixture of an aqueous solution of the immunostimulating substance such as norMDP, and the pharmaceutically acceptable oily substance. Accordingly, the immunization protocol advantageously provides separate injections of the immunogenic emulsion (immunogen) and the immunostimulating emulsion (supplement).

Alternatively, the improved method according to the invention provides effective neutralization of the subject animal's or patient's own hormones by immunization commencing with initial injections at separate sites. Thus, an anti-hormone immunizing emulsion mixture of an aqueous solution comprises an immunomimic antigen which is conjugated or fused with an immunogenic carrier, and a pharmaceutically acceptable oily substance. The second emulsion comprises a mixture of an immunostimulating substance dissolved in an aqueous vehicle, such as, e.g., norMDP, and a pharmaceutically acceptable oily substance. The systemically amenable oily substance is defined as Montanide ISA 703. Thus, one portion of the immunization means comprises the antigenic emulsion and the other portion comprises the supplement emulsion containing norMDP.

Another immunization method according to the invention provides concomitant, but separate intramuscular injections of the immunogenic emulsions and the immunostimulating emulsions at different sites of the same muscle group. For example, a preferred embodiment of the immunogen may comprise a GnRH peptide mimicking moiety containing the sequence of ten amino acids or fragment thereof, which is attached to a synthetic spacer peptide of 5-7 residues linked, bound complexed to an immunogenic carrier protein such, e.g. a diphtheria toxoid. The constructs are in accordance with those described in US 5,688,506, which disclosure is incorporated herein by

reference. For example, one embodiment comprising a conjugate, designated as D17-DT, is emulsified with Montanide ISA 703 to provide the innitial immunogenic injection preparation of this invention. Secondly, a supplementary injection preparation contains norMDP instead of the immunogenic conjugate or complex but is similarly emulsified in Montanide ISA 703.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 illustrates the increasing anti-GnRH antibody titer of groups of rabbits in response to a regimen of three separate injections of an immunogenic composition or vaccine and immunostimulating composition.

DETAILED DESCRIPTION OF THE INVENTION

It has been discovered that the method of the invention, namely, administering separate injections for the immunogenic emulsion and the immunostimulating emulsion, affords advantageously high antibody titers. According to this invention, therefore, the separate injections of immunogenic and immunostimulating portions provide an improved method of immunization over the conventional method which uses a formulation combining both portions in an injection. The immunization therapies which are particularly difficult to achieve are directed against an animal's including mammal's own antigens which are usually tolerated by the individual's own immune defense system capable of differentiating between "self" and "foreign" antigen. The inventive embodiment is particularly useful for immunizing a subject animal, such as mammal, including the human, in order to control or regulate intercellular and intracellular signals and events in the host.

The method improves the efficacy of immunization of a mammal, including the human, over the conventional vaccination methods by increasing the titer of specific antibodies against the subject's own antigens such as hormones and other factors, inter alia. Moreover, the separate administration of the primary and secondary portions of the vaccine potentially affords lower quantities and concentrations of antigen and inoculum and a reduced local reaction at the injection site.

One of the suitable embodiments of the invention is directed to a specific hormone vaccination, so as to achieve inhibition of said hormone, e.g., GnRH. The disclosures of U.S. Pat. Nos. 5,688,506 and 5,468,494 are incorporated herein by reference in their entirety.

For example, the hormone immunomimic portion of the immunogen comprises an epitope of the hormone. The function of the immunomimic molecular moiety of the immunogenic emulsion is intended to elicit antibodies crossreactive with the targeted hormone. Another suitable embodiment provides an immunogenic composition comprising a spacer element in the form of a peptide as a link through which the immunomimic peptide or other appropriate molecule is attached to an immunologically stimulating carrier, such as, e.g., a diphtheria toxoid ("DT"). Thus, the embodiment

generates the specific anti-hormone immune response. In one specific embodiment of the invention, the immunomimic peptide comprises the sequence of the C-17 peptide: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Ser-Ser-Pro-Pro-Pro-Pro-Cys (SEQ ID NO: 1 in the Sequence Listing of U.S. 5,688,506), or D-17 peptide: Cys-Pro-Pro-Pro-Pro-Ser-Ser-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (SEQ ID NO: 2 in the Sequence Listing of U.S. 5,688,506).

Other immunogenic carriers include tetanus toxoid, keylimpet hemocyanin, horseshoe crab hemocyanin, H. Pertussis, extract of Amercolata, bovine serum albumin, or ovalbumin. The immunogenicity can be further enhanced by the addition of toxoid adsorbing salts including, e.g., aluminum phosphate or aluminum potassium sulfate. The diphtheria toxoid and tetanus toxoid are toxins inactivated by formaldehyde fixation.

Example 1

The following example pertains to the immunization of female rabbits in accordance with the invention:

A novel immunization method has been applied such that the adjuvant Normuramyl Dipeptide (nMDP) is administered to the same muscle group receiving the GnRH immunogen but at a different site so as to enhance the antibody response with minimized local irritation. For this purpose, a sub-optimal dose of the D17DT conjugate (10 μg) was used. The immune response enhancing supplements containing doses of nMDP were given in amounts of 3 μg and 30 μg, which were dissolved and administered in PBS solution or in an emulsion with Montanide®ISA 703 at a second site from the immunogen. No nMDP was administered to negative control subjects, though a placebo was administered in the form of a PBS solution or a PBS and Montanide®ISA 703 emulsion as the supplement injection. In addition, conventional immunogens were formulated with the conjugate and nMDP combined into single injections in Montanide®ISA 703 emulsions. The nMDP was administered on all three dosing dates.

Test Materials of Example 1

(a) D17DT conjugate (Aphton).

(b) Montanide®ISA 703 (Seppic).

(c) Normuramyl Dipeptide (nMDP) (Peninsula Laboratories).

In accordance with the invention, three separate immunogen formulations were prepared as shown in Table 1. The D17-DT preparation was emulsified with the oily vehicle Montanide®ISA 703 (the immunogenic portion); the adjuvant nMDP was emulsified with Montanide®ISA 703 (the adjuvant supplement portion); and D17-DT in combination with nMDP was emulsified with Montanide®ISA 703 by the conventional method as described below.

Formulations of Example 1

TABLE 1					
Immunogen Formulations					
Immunogen	Vehicle	D17DT (mg/ml)	D17DT Dose in 0.2ml	nMDP (mg/ml)	nMDP Dose in 0.2ml
1	ISA 703	0.05	10μg	0	0
2	ISA 703	0.05	10μg	0.015	3 μg
3	ISA 703	0.05	10μg	0.15	30 μg

The immunogen emulsions were prepared under clean conditions as a 70:30 (wt:wt) ratio of vehicle to aqueous phase by mixing with the Silverson homogenizer (3 minutes at 8,000 rpm). The resultant emulsions were bottled in sterile multi-dose crimp cap serum vials, with individual vials filled for each dosing date. The materials were stored at 4°C and transported on cold packs.

TABLE 2			
Supplement Formulations			
Supplement Lot	Vehicle	nMDP (mg/ml)	Dose in 0.2 ml
A	PBS	0	0
B	PBS	0.015	3 μg
C	PBS	0.15	30 μg
D	ISA 703	0	0
E	ISA 703	0.015	3 μg
F	ISA 703	0.15	30 μg

Six formulations of supplement were prepared, as shown in Table 2. Two of these (A and D) were placebo controls; four of these (B, C, E and F) contained the adjuvant, nMDP. The supplementary formulations in PBS were prepared by dissolving the nMDP in PBS and filter-sterilizing (0.22 μm filter), then bottled in sterile multi-dose crimp cap serum vials, with individual vials filled for each dosing day. The supplement formulations comprising the Montanide®ISA 703 emulsions were prepared by the same procedures as the immunogenic emulsions, except that immunostimulatory nMDP was substituted for the conjugate in the PBS aqueous phase. The materials were stored at 4°C and transported on cold packs.

According to the these aforementioned protocol, the test immunizations were performed on day 0, 14, and 42 (see Schedule in Table 3 below). Female rabbits were used to perform the novel method of immunization for each of the comparative tests.

TABLE 3					
Rabbit Immunization Schedule					
Rabbit	Immunogen	Supplement	Injection 1	Injection 2	Injection 3

Group			Day 0	Day 14	Day 42
1	1	A	0.2ml each	0.2 ml each	0.2 ml each
2	1	B	0.2ml each	0.2 ml each	0.2 ml each
3	1	C	0.2ml each	0.2 ml each	0.2 ml each
4	1	D	0.2ml each	0.2 ml each	0.2 ml each
5	1	E	0.2ml each	0.2 ml each	0.2 ml each
6	1	F	0.2ml each	0.2 ml each	0.2 ml each
7	1	None	0.2 ml	0.2 ml	0.2 ml
8	2	None	0.2 ml	0.2 ml	0.2 ml
9	3	None	0.2 ml	0.2 ml	0.2 ml

The immunizations were performed on 5 rabbits for each subject test group for a total of 45 female rabbits. The bleed schedule provided for a bleeding to be done every 14 days from day 0 through day 68.

The sera were prepared and stored frozen according to standard procedures.

Concerning the dosing procedures, each rabbit in Groups 1 through 6 received two (2) injections on each dosing day, including one (1) injection of Immunogen and one (1) injection of Supplement on the scheduled three (3) dosing days. Thus the combined total number of injections per rabbit of Groups 1 through 6 were three (3) of Immunogen and three (3) of Supplement. Animals in Groups 7 through 9 each received one (1) injection of Immunogen on the scheduled three (3) dosing days, wherein the adjuvant was admixed to the immunogen for a single injectable preparation. The combined total number of injections per rabbit in these groups was three (3) of Immunogen.

The injection doses consisted of 0.2 ml/injection (for each Immunogen, Supplement, and Mix) on each of the three dosing days. After the formulations were allowed to equilibrate to room temperature for 1 hour, each formulation was vigorously shaken prior to dispensing. As indicated above, the dosing schedule provided that the doses 1, 2 and 3 were given on days 0, 14, and 42, respectively. The injection route was intramuscularly (IM) in the hind leg. Thus, the first and second doses were administered to the same leg, and the third dose was administered to the other leg. The nMDP supplement portion was injected as close to the hip (but in the hindleg muscle) as possible, at least 3-4 cm above the site at which the anti-GnRH immunogen portion of the vaccine was injected. Each injection was administered at a muscle site that had not previously been injected. The injection sites were tattooed so as to identify them later.

The Antibody Assay

The sera were tested by an ELISA specifically designed to detect anti-GnRH antibodies. The results of the study of Example 1 showed that the separate injections of immunogen emulsion and the supplement (nMDP adjuvant) emulsion afforded a surprisingly advantageous method for the effective

raising of the anti-hormonal antibody titer in the subject animals. For the purpose of clearly determining the improvement effect of this method suboptimal amounts of the antigen were used.

It has been discovered that the immunizing effect of the suboptimal amounts of antigen, i.e., 10 μg of D17DT in Montanide®ISA 703, was much enhanced by the second and third booster injections of the supplement emulsions in the amounts of 3 or 30 μg nMDP in Montanide®ISA 703. For example, after 68 days, that is about 4 weeks after the last (third) dosing, the anti-GnRH antibody titer rose to about 12,500 when using separate emulsions of 10 μg D17-DT/ISA 703 and 3 μg nMDP/ISA 703, respectively, and to about 26,000 when using similarly 10μg D17-DT/ISA 703 and 30 μg nMDP/ISA 703, respectively. Animals that received the D17-DT antigen and nMDP supplement combined (Immunogen 2 and 3) in a single Montanide®ISA 703 injection responded with antibody titers similar to these groups. Thus, administration of nMDP as a supplement in Montanide®ISA 703 (group 5 and 6) was equally as effective as mixing the nMDP with the conjugate in a single formulation (group 8 and 9). Moreover, antibody titers were about 2 to 4 fold higher than, for example, the titers observed for dosings of an emulsion with 0 μg nMDP indicating the immunopotentiating effect of the supplemental nMDP.

Surprisingly, Groups 1, 2 and 3, which received adjuvant (nMDP) in the form of a PBS solution, did not produce effective levels of anti-GnRH antibody. Thus the nMDP supplement was effective when given as a sustained release emulsion formulation (such as in Montanide®ISA 703) not as an aqueous solution.

The antibody titer assays of the immunized female rabbits are tabulated in Table 4 below.

TABLE 4		Antibody Titers					
Group #	Rabbit #	Day 0	Day 14	Day 28	Day 42	Day 56	Day 68/70
1	1	0	0	132	746	2,878	3,708
	2	0	11	1,388	1,035	2,813	2,757
Immunogen:10µg D17DT in ISA 703	3	0	847	4,211	5,343	12,400	27,600
Supplement:PBS only (Control)	4	0	0	276	1,019	5,940	6,513
	5	0	125	1,090	1,259	2,349	2,118
	Mean	**0**	**34**	**722**	**1,015**	**3,495**	**3,774**
	Median	**0**	**6**	**683**	**1,027**	**2,846**	**3,233**
	S. D.	**0**	**61**	**613**	**230**	**1,647**	**1,939**
2	6	0	0	205	88	957	1310
	7	0	0	567	261	2084	1865
Immunogen:10µg D17DT in ISA 703	8	0	225	432	946	3251	3160
Supplement:3µg nMDP in PBS	9	0	0	360	864	1988	2352
	10	0	402	3,096	2338	4058	3979
	Mean	**0**	**125**	**932**	**899**	**2,468**	**2,533**
	Median	**0**	**0**	**432**	**864**	**2,084**	**2,352**
	S. D.	**0**	**183**	**1,217**	**886**	**1,204**	**1,056**
3	11	0	0	229	547	2186	1962
	12	0	205	1,075	664	1511	1815
Immunogen:10µg D17DT in ISA 703	13	0	0	234	253	984	1709
Supplement: 30 µg nMDP in PBS	14	0	251	348	431	1660	2836
	15	0	55	678	726	1326	4789
	Mean	**0**	**114**	**472**	**474**	**1,585**	**2,031**
	Median	**0**	**103**	**291**	**489**	**1,586**	**1,889**
	S. D.	**0**	**133**	**406**	**176**	**494**	**514**
4	16	0	456	1,741	1014	1828	1343
	17	0	1,524	1,079	1439	3045	1918
Immunogen:10µg D17DT in ISA 703	18	0	319	590	772	940	1407
Supplement: ISA 703 only (Control)	19	0	119	513	654	2703	2681
	20	0	65	623	669	1840	1959
	Mean	**0**	**497**	**909**	**910**	**2,071**	**1,862**
	Median	**0**	**319**	**623**	**772**	**1,840**	**1,918**
	S. D.	**0**	**595**	**515**	**329**	**828**	**538**
5	21	0	152	549	646	1291	1150
	22	0	1,226	2,126	2773	2092	2133
Immunogen:10µg D17DT in ISA 703	23	0	683	2,529	6095	6781	7199
Supplement: 3 µg nMDP in ISA 703	24	0	1,795	13,700	21,600	27,400	30,500
	25	0	2,545	13,300	13,300	21,800	21,500
	Mead	**0**	**1,280**	**6,441**	**8,883**	**11,873**	**12,496**
	Median	**0**	**1,226**	**2,529**	**6,095**	**6,784**	**7,199**
	S. D.	**0**	**935**	**6,488**	**8,576**	**11,971**	**12,936**
6	26	0	1,286	5,777	13,000	21,300	10,300
	27	0	539	8,749	16,100	32,800	20,900
Immunogen:10µg D17DT in ISA 703	28	0	1,673	12,000	15,700	17,900	13,100
Supplement: 30 µg nMDP in ISA 703	29	0	1,384	10,400	10,500	20,700	22,300
	30	0	1,360	6,838	22,300	37,400	21,000
	Mean	**3**	**1,248**	**8,753**	**15,520**	**26,020**	**17,580**
	Median	**0**	**1,360**	**8,749**	**15,700**	**21,300**	**20,900**
	S. D.	**6**	**423**	**2,539**	**4,413**	**8,544**	**5,373**
7	31	0	152	1,796	3885	5998	8827
	32	0	77	819	2559	5915	8874
10 µg D17DT+ 0 nMDP	33	0	948	728	1231	3816	6869
Single Injection in ISA 703	34	0	127	918	981	3358	4241
	35	0	0	481	5913	1395	3549
	Mean	**0**	**255**	**948**	**2,914**	**4,096**	**6,472**
	Median	**0**	**127**	**819**	**2,559**	**3,816**	**6,869**
	S. D.	**0**	**375**	**501**	**2,039**	**1,926**	**2,500**

Group #	Rabbit #	Day 0	Day 14	Day 28	Day 42	Day 56	Day 68/70
8	36	0	1,150	12,000	46,800	28,800	33,100
	37	0	1,014	6,331	12,300	9132	9463
10 µg D17DT + 3µg nMDP	38	0	816	5,025	5886	5406	8625
Single Injection in ISA 703	39	0	157	5,401	7667	8315	8067
	40	0	0	5,720	12,200	10,800	10,700
	Mean	0	497	5,619	9,513	8,413	9,214
	Median	0	487	5,561	9,934	8,724	9,044
	S. D.	0	494	553	3,243	2,256	1,145
9	41	0	1,153	16,500	27,200	50,300	48,100
	42	0	1,765	28,500	27,700	16,300	15,000
10 µg D17DT + 30 µg nMDP	43	0	366	5,496	12,700	16,000	23,800
Single Injection in ISA 703	44	0	360	12,000	19,000	10,100	9,018
	45	0	575	11,800	29,000	17,200	13,100
	Mean	0	844	14,859	23,120	21,980	21,884
	Median	0	575	12,000	27,200	16,300	15,000
	S. D.	0	608	8,573	7,031	16,078	15,660

Gross Pathology Observations

The injection sites were evaluated for gross (macroscopic) appearance of the thigh muscle after injection of test materials. Mean gross pathology scores by group are presented in Table 5. The separate injections of antigen and adjuvant were well tolerated (Groups 1-6), with scores ranging from normal tissue to minimal pathology present. A comparison of injection site reactions on groups paired for dose of adjuvant (Groups 4 vs. 7, 5 vs. 8, 6 vs. 9), indicates that mixing the antigen and adjuvant in a single injection resulted in significantly higher irritation or pathology scores than seen in the corresponding supplement groups. Therefore, separate administration of the nMDP adjuvant led to a marked improvement in injection site tolerance of the immunogen.

TABLE 5

Mean Gross Pathology Scores

Group #	Immunogen Site #1	Supplement Site #1	Immunogen Site #2	Supplement Site #2	Immunogen Site #3	Immunogen Site #3
1	0	0	0	0	0	0
2	0	0.25	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0.25	0	0.75	0.25
5	0	0	0.25	0	0	0.25
6	0.5	0.25	0.25	0.5	1.0	0.5
7	0.25	N/A	0.5	N/A	0.75	N/A
8	0.5	N/A	1.5	N/A	2.0	N/A
9	1.0	N/A	2.5	N/A	3.0	N/A

0—Normal Tissue
1—Minimal Pathology
2—Moderate Pathology
3—Severe Pathology

Intermediate grades are assigned when lesions do not fall unequivocally within the definition of a certain grade.

The above example demonstrates that the present invention provides an improved method of immunization both by increasing the immune response of an immunized animal in terms of producing antibodies, and by reducing undesirable injection site reactions. The method comprises a separate immune response-stimulating composition containing a nontoxic adjuvant, which is administered separately from the actual immunogen. The immunogen itself can be constructed to target the immune response against the effective (i.e., accessible) epitopes of pathogenic organisms as well as other antigens of normal and malignant tissue or cells. One skilled in the art will recognize that the separate steps of immunization of this invention would afford a wide variety of applications and strategies so as to significantly improve the therapeutic success of immunization.

These results are significant, as the capacity to selectively administer supplemental adjuvant affords the physician with additional control over the immunization treatment. This is advantageous, as the principal benefit of nMDP enhancement appear to be expressed in the primary injection (separate study from those described here) when optimal doses are administered; hence, nMDP may not be needed in subsequent injections. Thus, the physician can elect to administer the adjuvant if it is needed to boost the response in a patient with suboptimal responsiveness, as well as to decide upon the optimal dose of adjuvant to administer with the selected dose of vaccine. This would not be an option with the conventional approach, wherein the nMDP is formulated with the conjugate (antigen) at the time of manufacture. Moreover, at lower dosages of conjugate, the formulations are better tolerated, despite being equally immunogenic. Thus, this invention enables the immunization regimen to be tailored to best suit the needs of the individual patient.

Whereas the present examples pertain to the use of water-in-oil emulsions, the skilled formulator would expect that oil-in-water emulsions are also applicable.

In addition to Montanide ISA 703, other metabolizable oily substances such as Montanide ISA 719 as well as Montanide ISA 720 provide stable water-in-oil (70:30 or 50:50) emulsions. An oil-in-water emulsion can be produced by mixing 25 parts of Montanide ISA 25 oily vehicle with 75 parts of aqueous phase.

In addition to the above described examples, the advantageous invention can be envisioned even by one of ordinary skill to encompass effective immunization of animals, in particular, mammals including humans, in defense against various organisms or control of physiological activities by the many hormones, factors, and receptors involved with the normal and abnormal intercellular regulation.

For example, the advantageous invention can be directed to immunizing against gastrin or the

cleaved gastrin peptides (G17 and G34), in accordance with the above-referenced coassigned U.S. Patents No. 5,023,077 and 5,468,494, which are incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. A method for immunization comprising separately administering an immunogenic sustained release composition and an immune response enhancing composition.

2. The method of claim 1, wherein the secondary composition comprises a compound which is effective in stimulating a strong immune response.

3. The method of claim 1, wherein the method elicits a significant anti-immunogen antibody titer increase.

4. The method of claim 1, wherein the immunogenic composition comprises a pharmaceutically acceptable immunogenic emulsion comprising an antigen which comprises an immunomic domain and an immunogenic domain.

5. The method of claim 1, wherein the separate steps of immunizing are administered at an initial dosing and subsequent booster dosings.

6. The method of claim 1, wherein the immunogenic composition comprises a conjugate or complex of an immunomimic domain with an immunogenic carrier.

7. The method of claim 1, wherein the immunogenic composition and the immune response enhancing composition each comprise a pharmaceutically acceptable oily substance.

8. The method of immunization of claim 6, wherein the immunomimic domain comprises an epitope selected from the group consisting of a microbe protein, an autologous eukaryotic cell antigen, an heterologous eukaryotic cell antigen, an enzyme, a cofactor, and a hormone.

9. The method of immunization of claim 7, wherein the oily substance comprises Montanide ISA 703, Montanide ISA 25, Montanide ISA 719, or Montanide ISA 720.

10. The method of claim 1or 2, wherein the immune response enhancing composition comprises an immunostimulating compound.

11. The method of claim 10 wherein the immunostimulating compound is formulated for sustained release.

12. The improved method for immunization of claim 11, wherein the sustained release formulations further comprise liposomes, microparticles, and implantable vehicles for delivery.

13. The method of claim 10, wherein the immunostimulating compound is norMDP, threonyl MDP, LPS, or SLPS.

14. An immunization kit for increasing the immune response to a vaccine target comprising, in separate sustained release preparations,

(i) an immunogenic composition, and

(ii) an immune response stimulating composition.

15. The immunization kit of claim 12, wherein the immunogenic composition comprises an

epitope of an immunogen target.

16. The immunization kit of claim 12, wherein the immune response stimulating composition comprises an effective, nontoxic adjuvant.

17. The immunization kit of claim 12, wherein the compositions are kept in separate containers for separate inoculations.

18. The immunization kit of claim 12, wherein the sustained release preparations comprise emulsions, liposomes, microparticles, or implantable vehicles.

19. An improved composition for parental immunization comprising separately (i) a formulation for a sustained release immunogenic composition and (ii) an immune response enhancing composition.

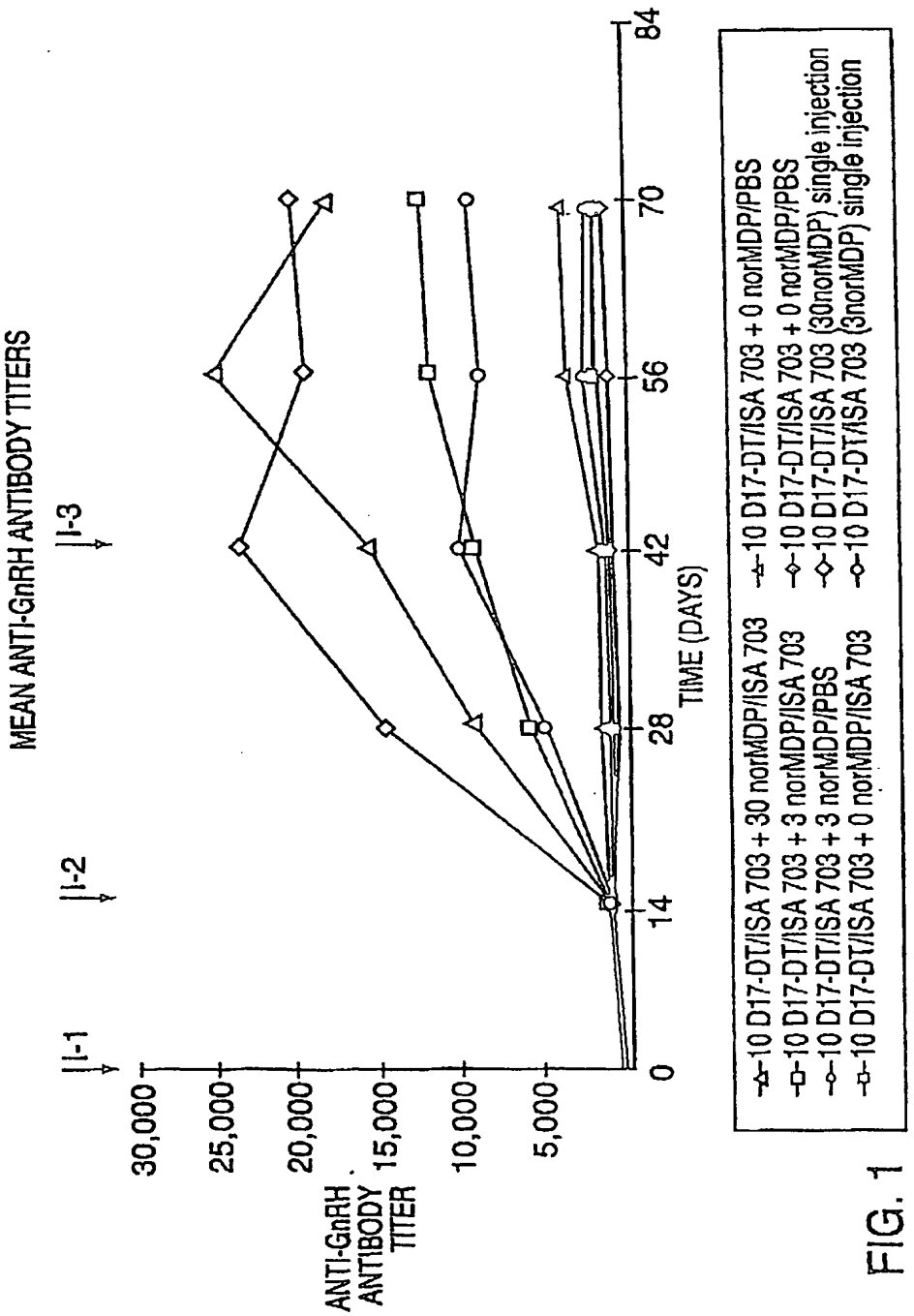
MEAN ANTI-GnRH ANTIBODY TITERS
I-1
I-2
I-3
30,000
25,000
20,000
15,000
10,000
5,000
ANTI-GnRH ANTIBODY TITER
0
14
28
42
56
70
84
TIME (DAYS)
10 D17-DT/ISA 703 + 30 norMDP/ISA 703
10 D17-DT/ISA 703 + 3 norMDP/ISA 703
10 D17-DT/ISA 703 + 3 norMDP/PBS
10 D17-DT/ISA 703 + 0 norMDP/ISA 703
10 D17-DT/ISA 703 + 0 norMDP/PBS
10 D17-DT/ISA 703 (30norMDP) single injection
10 D17-DT/ISA 703 (3norMDP) single injection

FIG. 1

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(74) Agents: **DRIVAS, Dimitrios, T.** et al.; White & Case LLP, Patent Department, 1155 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

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(54) Title: IMPROVED METHOD OF IMMUNIZATION

(57) Abstract: A method for improving the immune response to a immunogen by separately administering to a patient an immunogen composition for sustained release comprising an epitope of the immunogen target, and a supplement comprising an adjuvant compound for stimulating, potentiating or activating a strong immune response. The provided method potentially reduces local irritation at the sites of inoculation, i.m. or s.c.

INTERNATIONAL SEARCH REPORT

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PCT/US 00/30778

A. CLASSIFICATION OF SUBJECT MATTER
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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, PAJ, WPI Data

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Noë, V

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(74) Agents: DRIVAS, Dimitrios, T. et al.; White & Case LLP, Patent Department, 1155 Avenue of the Americas, New York, NY 10036 (US).

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(54) Title: IMPROVED METHOD OF IMMUNIZATION

(57) Abstract: A method for improving the immune response to a immunogen by separately administering to a patient an immunogen composition for sustained release comprising an epitope of the immunogen target, and a supplement comprising an adjuvant compound for stimulating, potentiating or activating a strong immune response. The provided method potentially reduces local irritation at the sites of inoculation, i.m. or s.c.

IMPROVED METHOD OF IMMUNIZATION

The present application claims priority from the Provisional U.S. Application 60/164,054 filed November 8, 1999.

FIELD OF INVENTION

The invention relates to an improved method of immunization, and in particular, to a method of preparing an immunizing composition for separate injection of an immunogenic portion of and an adjuvant portion of the composition.

BACKGROUND OF INVENTION

Antigenic compositions or vaccines are formulated to be effective in immunizing an animal or a human against invading foreign organisms or matter or infective agents by eliciting an immune response which culminates, inter alia, in the production of antibodies directed against the invading organisms or matter or infective agents. Thus, the compositions elicit antibodies that are specifically directed against the antigenic aspects of the foreign substances or microorganism or other infective agents, including pathogenic organisms, toxic products of said organisms, and viruses.

The success of an antigenic composition is linked to its immunogenicity, that is, the ability to produce a sufficiently high or effective titer of antibodies to bind to the target pathogen or pathogenic toxins. Thus, the immunogenicity depends on the effectiveness by which the antigen causes the body's immune system to elicit a response. The immune response is generally assessed on the basis of the antibody titer in the blood of the immunized animal or mammal including the human. Immunizing formulations targeted against antigens of low immunogenicity are usually constructs or mixtures of the select immunomimic epitope of a target antigen and an immunogenic carrier component not related to the target antigen. The immunogenic component aids or causes a strong immune response against the entire construct or mixture so as to be effective against the target antigen. Supplemental components, in the context of the invention, are adjuvants, such as, e.g., bacterial extracts or specific synthesized products.

Thus, the antibodies elicited by the antigenic formulation can bind to specific targets on the cell surfaces or free circulatory proteins. Specific antibody populations can act as biological modifying or interfering agents affecting the physiological activities of the body, the cells tissues or by binding to receptors, enzymes, signal transducing peptides or other proteins. Cell cycle control or checkpoints and numerous biological activities or events can be affected by the specifically targeted antibodies.

In order to further enhance or potentiate the immune defense system, so-called adjuvants in the form of oily substances and other potentiating agents are conventionally added to the antigenic formulations. Adjuvants are usually mixed into the immunogenic formulation and simultaneously delivered with the antigen in the same administration, e.g., by injection. In particular, the formulations have been enhanced to raise effective antibody titers against fractions of microorganisms or viral pathogens by the addition of so-called adjuvants. Adjuvants are compositions comprising immune response-stimulating killed microbial cells, particles, fragments or components thereof. Moreover, immunogenic compositions can contain different kinds of carrier, including emulsions, liposomes, microparticles and implantable vehicles which are, moreover, metabolizable.

More recently developed anti-infective agent immunization technology as described above, has been applied as a modifying biological means to immunize against various soluble and insoluble animal, including human, antigens normally not recognized or targeted by the individual's own immune defense, but which can be rendered immunogenic so as to significantly stimulate or potentiate the individual's own immune response system against itself. The self-antigens can include the surfaces of certain cells which are dysfunctional or malignant, and small proteins, enzymes or intercellular signals, such as, e.g., hormones or other signal factors. The lack of effective immunogenicity of these self-antigens can often be overcome by complexing or linking the non-immunogenic self-antigens with a pharmaceutically acceptable, i.e. non-toxic, immunogenic carrier so as to produce antibodies capable of binding, thereby neutralizing, the self-antigen epitope of the subject animal or human patient.

Immunization against hormones in mammalian and, in particular, human subjects, has been therapeutically applied to control or modify hormonal effects on the physiology of the body. In many cases, as disclosed e.g., in U.S. Patent Nos. 5,688,506, 4,526,716, 4,676,981, 5,023,077, 5,891,792, 5,843,446, and 5,723,129, the immunization procedure requires repeated injections of formulations containing an immunogen and an immune response stimulating adjuvant.

The immunomimic portion of the immunogen construct can encompass epitopes of both a non-peptide and peptide variety so that the immune reaction is directed to the structural and/or functional properties of the target hormones or other factors. The immunological methods for the therapeutical hormone action control or modification can be used for the treatment of patients afflicted with a disorder or disease. For example, the immunological method for the control of a peptide hormone can control certain physiological activity in normal or disease-afflicted subjects.

Some immunogenic constructs suitable for hormone-controlling or modifying effect comprise immunomimicking molecular moieties of the hormones which are conjugated or fused to immunogenic carriers, which may comprise complex polysugars, proteins or peptides. The immunogenic constructs are usually administered in the form of an emulsion, either an oil-in-water or a water-in-oil emulsion, containing an adjuvant capable of stimulating or potentiating an immune response. A conventional methodology includes mixing the preparation of an antigenic emulsion compositions. For example, a water-in-oil emulsion is usually prepared by mixing equal parts or ratios of 70:30 of an aqueous solution of an antigen with Freund's Complete Adjuvant containing inactivated mycobacteria in a suitable oily substance (i.e., mineral oil). Since the Freund's Complete Adjuvant is very irritating in the human subject, pharmaceutically more amenable oily substances other than mineral oil have come into use. Acceptable oil phase vehicles have been selected from metabolizable oily substances.

An immune response is typically measured in terms of the production of specific antibodies. For example, the hormones which are targeted for control by the immunological methods are directly neutralized by the antigen-binding reaction with circulating specific antibodies elicited by the immunogenic constructs. Other affected hormones, forming cascades of hormonal activities upon the signal of the primary hormone.

An anti-hormone immunogen has been constructed to affect the regulation of the gonadotropin releasing hormone (co-assigned U.S. Patent 5,688,506). The Gonadotropin Releasing Hormone (abbreviated "GnRH", also known as Luteinizing Hormone Releasing Hormone, abbreviated "LHRH"), is of central importance to the regulation of fertility. Johnson M et al., *Essential Reproduction*, 3rd Edn. Blackwell Scientific Publications (1988). In both males and females, GnRH is released from the hypothalamus into the bloodstream and is transported through the bloodstream to the pituitary, where it induces the release of gonadotropins, luteinizing hormone and follicle stimulating hormone, by the gonadotrophs. These gonadotropins, in turn, act upon the gonads, inducing steroidogenesis and gametogenesis. Steroids released from the gonads into the circulation subsequently act upon various tissues. This gonadotropin related hormonal cascade can be halted by neutralization of the biological activity of GnRH. Fraser H.M., Physiological Effects of Antibody to Lutenizing Hormone Releasing Hormone, *Physiological Effects of Immunity Against Reproductive Hormones*, Edwards and Johnson, Eds. Cambridge University Press (1976). As a consequence of GnRH neutralization, the gonadotropins and gonadal steroids are not released into the blood, and their biological activities are curtailed or eliminated by the direct and indirect action of specific anti-GnRH

antibodies. By eliminating the physiological activity of GnRH, the cascade of hormonal regulation of fertility is interrupted and gametogenesis ceases. Consequently, GnRH neutralization halts the production of gametes. Thus, GnRH neutralization is an effective means of contraception.

Furthermore, a number of important diseases are affected by gonadotropins and particularly gonadal steroid hormones. Such diseases include breast cancer, uterine and other gynecological cancers, endometriosis, uterine fibroids, benign prostatic hypertrophy and prostate cancer, among others. Removal of the gonadal steroid hormonal stimuli for these diseases constitutes an important means of therapy. An effective method of accomplishing this is by immunologically neutralizing GnRH, to thereby eliminate gonadal steroids that induce and stimulate these diseases. McLachlan R.I. et al. Clinical Aspects of LHRH Analogues in Gynaecology: a Review, *British Journal of Obstetrics and Gynaecology*, 93:431-454 (1986); Conn P.M. et al. Gonadotropin-Releasing Hormone and Its Analogs, *New England Journal of Medicine*. 324:93-103 (1991) and Filicori M. GnRH Agonists and Antagonists, Current Clinical Status. *Drugs*. 35:63-82 (1988).

Since GnRH has the same amino acid sequence in all mammals (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2), it is presumed that a single immunogen would be effective in all mammalian species, including humans. The so-called active form of immunization has required a composition of immunogens against GnRH which elicits a sufficient titer of anti-GnRH antibodies in the patient to physiologically neutralize and thereby limit the hormone level in the patient.

Thus, the anti-GnRH directed conjugate of certain analogous peptides to the GnRH immunomimic domain can be linked to a carrier protein which is effectively immunogenic, such as, e.g., diphtheria toxoid, tetanus toxoid, keylimpet hemocyanin, bovine serum albumin, Hemophilae pertussis extracts or filamentous Amycolata extracts. Consequently, the immune response to GnRH-vaccine will be primarily directed against the carrier protein and secondarily, the attached hormone epitope moiety (hapten).

As previously discussed, the Freund's Complete Adjuvant is accepted as an unofficial standard of efficacy for inducing an immune response. However, it is not suitable for use in humans since it is very reactogenic and irritating. Amenable adjuvants, therefore, have been chosen from non-toxic tissue-amenable compounds or compositions, such as, e.g., norMDP or MDP. However, even with acceptable adjuvants, the inoculations often cause a local inflammatory reaction which can be very discomforting or painful to the patient. Tissue reactogenicity is a particularly undesirable side effect when the administration of the vaccine must

be repeated in order to maintain or boost the antibody titer, often leading to more severe irritation of the injection sites potentially resulting in painful lesions. Moreover, in order to obtain the desired level of the immune response to an immunogenic construct, a relatively large amount of adjuvant is sometimes required, thereby often aggravating the local tissue reaction of both i.m. and s.c. injection site. Finally, the accompanying tissue reaction of a large inoculum can lead to limited immune response to the target antigen.

Therefore, it is an object of this invention to provide delivery of the adjuvant portion of an immunogenic composition separately from the immunogen, while providing adjuvant driven enhancement of the immune response against the antigenic domains or components of the immunogenic composition. In particular, it is an object of this invention to increase the efficacy of immunization, by an improved procedure which has the potential to reduce local irritation and other side effects without loss of efficacy. It is a further object of the invention to provide separate preparations of immunogen and supplement for immunization against hormonal or non-hormonal antigens.

SUMMARY OF THE INVENTION

The present invention is directed to an improved method for the immunization of an animal or mammal including the human whereby the local tissue irritation of the injection site may be reduced while the immune response is advantageously enhanced.

The improved method has been surprisingly effective in enhancing a high antibody titer, such that the amount of immunomimicking portion of the immunogen can be reduced resulting in more gentle and economic immunization treatments as well as other advantages affecting the immune response.

According to the invention, the improved method comprises the separate administration of an immunogenic portion comprising the immunogen and a potentiating supplement portion comprising the immunostimulating additive or adjuvant.

Thus, with the invention, a single adjuvant formulation can be used to enhance the immune response to each of many different immunogens. Moreover, the dose of adjuvant can be selectively varied for a specific application of each immunogen which enables the use of a single immunogenic formulation with selected administration of a separate adjuvant. A further advantage of the present invention is found in separate administration steps to help overcome any incompatibility between adjuvant and immunogen formulation components.

Another embodiment according to the invention provides the selection of different adjuvants for each dosing of a immunizing composition. Separate administration of adjuvants also

affords formulation of adjuvant compositions incorporating suitable characteristics for *in vivo* particle release rates different from the immunogenic components. Consequently, the adjuvant driven enhancement or amplification of the immune response can be optimized for each immunization step.

Another advantage of the present invention can be found in that adjuvants can be administered for selected administrations of immunogenic constructs in order to optimize the immunopotentiation of the antibody response.

In addition, the adjuvant formulations for the separate administration, as provided by this invention, comprise sustained release formulations, including emulsions, liposomes, microparticles and implantable vehicles, as are known in the pharmacological art.

The immune response can be further manipulated by varying the timing of adjuvant supplement administration with regard to the immunization protocol. In particular, the adjuvant compositions can be administered at suitable anatomical sites located near or distant from the immunization site itself.

Furthermore, immunostimulating adjuvants can be administered by different routes so as to optimize the immunopotentiating activity of the adjuvant for a given immunogen. In particular, the routes of administering an adjuvant to a patient in need thereof can be identical or different from the immunization. Thus, the separate injections of immunogenic composition and adjuvant composition can be located in close proximity adjacent to each other, in accordance with an embodiment of this invention.

According to invention, the separately administered immunizing portions comprise firstly a primary emulsion containing the immunogenic portion (also referred to as the immunogen) and secondly, a secondary emulsion containing an immunostimulating adjuvant (also referred to as the supplement).

The present invention provides separate administrations of biocompatible adjuvants which can be muramyl dipeptide (MDP) derivatives, including norMDP and threonyl MDP. Other suitable adjuvant preparations can utilize lipopolysaccharide (LPS) derivatives, including soluble lipopolysaccharide (SLPS) for use in separate adjuvant administrations. Dependent on the chemical or physical nature of the adjuvants, the formulations for separate administrations may include aqueous solutions, which can contain sterile water, saline or phosphate buffered saline. Preferably, the aqueous solution are neutral isotonic compositions.

In accordance with the present invention, a vaccination kit can be provided for increasing the immune response to a target vaccine wherein the kit comprises in separate aseptic

preparations or entities, an immunogenic composition and an immune response stimulating or potentiating composition. The preparations can be provided under aseptic conditions.

Another embodiment according to the invention provides an improved method for effectively modifying, controlling, or inhibiting the physiological function of a mammalian or human hormone by separately administering an immunizing formulation comprising an anti-hormone immunogenic construct and another kind of vaccine formulation comprising an immune response stimulating additive or adjuvant. The separate administration of the supplement is provided so as to reduce or avoid side reactions such as severe tissue reaction or inflammation while enhancing the immune response to the immogen. The invention, therefore, provides that the primary part of the immunogenic water-in-oil emulsion contains an anti-hormone immunogenic conjugate and a secondary part of the water-in-oil vaccine emulsion contains a preferred norMDP adjuvant or supplement. The immunogenic construct portion is administered at a site which is administered to the same tissue group or organ but is separate from the inoculation site of the supplement comprising the immunostimulating or immunopotentiating adjuvant emulsion. The novel method of separate injections is thus providing an immunization more effective for generating an immune response and potentially less stressful for the subject animal or patient.

In accordance with another embodiment of the invention, the initial immunogen or vaccine can be delivered by conventional methods, namely, by a single parenteral injection of an emulsion mixture of the immunogenic conjugate, which also contains an immunostimulating additive, such as, e.g., norMDP, and the oily substance acceptable for human use as, e.g., Montanide ISA 703, Montanide 25, Montanide ISA 719, or Montanide ISA 720 (Seppic). The initial immunization step is, however, followed by booster injections at two separate locations on the suitable tissue. The booster injections comprise an immunogenic emulsion prepared from a mixture of an aqueous solution of an immunomimic antigen, which is linked to an immunogenic carrier, and emulsified with a pharmaceutically acceptable oily substance such as e.g., Montanide ISA 703, Montanide ISA 719 or Montanide ISA 720. The second or supplement emulsion comprises a mixture of an aqueous solution of the immunostimulating substance such as norMDP, and the pharmaceutically acceptable oily substance. Accordingly, the immunization protocol advantageously provides separate injections of the immunogenic emulsion (immunogen) and the immunostimulating emulsion (supplement).

Alternatively, the improved method according to the invention provides effective neutralization of the subject animal's or patient's own hormones by immunization commencing with initial injections at separate sites. Thus, an anti-hormone immunizing emulsion mixture of an

aqueous solution comprises an immunomimic antigen which is conjugated or fused with an immunogenic carrier, and a pharmaceutically acceptable oily substance. The second emulsion comprises a mixture of an immunostimulating substance dissolved in an aqueous vehicle, such as, e.g., norMDP, and a pharmaceutically acceptable oily substance. The systemically amenable oily substance is defined as Montanide ISA 703. Thus, one portion of the immunization means comprises the antigenic emulsion and the other portion comprises the supplement emulsion containing norMDP.

Another immunization method according to the invention provides concomitant, but separate intramuscular injections of the immunogenic emulsions and the immunostimulating emulsions at different sites of the same muscle group. For example, a preferred embodiment of the immunogen may comprise a GnRH peptide mimicking moiety containing the sequence of ten amino acids or fragment thereof, which is attached to a synthetic spacer peptide of 5-7 residues linked, bound complexed to an immunogenic carrier protein such, e.g. a diphtheria toxoid. The constructs are in accordance with those described in US 5,688,506, which disclosure is incorporated herein by reference. For example, one embodiment comprising a conjugate, designated as D17-DT, is emulsified with Montanide ISA 703 to provide the initial immunogenic injection preparation of this invention. Secondly, a supplementary injection preparation contains norMDP instead of the immunogenic conjugate or complex but is similarly emulsified in Montanide ISA 703.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 illustrates the increasing anti-GnRH antibody titer of groups of rabbits in response to a regimen of three separate injections of an immunogenic composition or vaccine and immunostimulating composition.

DETAILED DESCRIPTION OF THE INVENTION

It has been discovered that the method of the invention, namely, administering separate injections for the immunogenic emulsion and the immunostimulating emulsion, affords advantageously high antibody titers. According to this invention, therefore, the separate injections of immunogenic and immunostimulating portions provide an improved method of immunization over the conventional method which uses a formulation combining both portions in an injection. The immunization therapies which are particularly difficult to achieve are directed against an animal's including mammal's own antigens which are usually tolerated by the individual's own immune defense system capable of differentiating between "self" and "foreign" antigen. The inventive embodiment is particularly useful for immunizing a subject animal, such as

mammal, including the human, in order to control or regulate intercellular and intracellular signals and events in the host.

The method improves the efficacy of immunization of a mammal, including the human, over the conventional vaccination methods by increasing the titer of specific antibodies against the subject's own antigens such as hormones and other factors, inter alia. Moreover, the separate administration of the primary and secondary portions of the vaccine potentially affords lower quantities and concentrations of antigen and inoculum and a reduced local reaction at the injection site.

One of the suitable embodiments of the invention is directed to a specific hormone vaccination, so as to achieve inhibition of said hormone, e.g., GnRH. The disclosures of U.S. Pat. Nos. 5,688,506 and 5,468,494 are incorporated herein by reference in their entirety.

For example, the hormone immunomimic portion of the immunogen comprises an epitope of the hormone. The function of the immunomimic molecular moiety of the immunogenic emulsion is intended to elicit antibodies crossreactive with the targeted hormone. Another suitable embodiment provides an immunogenic composition comprising a spacer element in the form of a peptide as a link through which the immunomimic peptide or other appropriate molecule is attached to an immunologically stimulating carrier, such as, e.g., a diphtheria toxoid ("DT"). Thus, the embodiment generates the specific anti-hormone immune response. In one specific embodiment of the invention, the immunomimic peptide comprises the sequence of the C-17 peptide: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Ser-Ser-Pro-Pro-Pro-Pro-Cys (SEQ ID NO: 1 in the Sequence Listing of U.S. 5,688,506), or D-17 peptide: Cys-Pro-Pro-Pro-Pro-Ser-Ser-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (SEQ ID NO: 2 in the Sequence Listing of U.S. 5,688,506).

Other immunogenic carriers include tetanus toxoid, keylimpet hemocyanin, horseshoe crab hemocyanin, H. Pertussis, extract of Amercolata, bovine serum albumin, or ovalbumin. The immunogenicity can be further enhanced by the addition of toxoid adsorbing salts including, e.g., aluminum phosphate or aluminum potassium sulfate. The diphtheria toxoid and tetanus toxoid are toxins inactivated by formaldehyde fixation.

Example 1

The following example pertains to the immunization of female rabbits in accordance with the invention:

A novel immunization method has been applied such that the adjuvant Normuramyl Dipeptide (nMDP) is administered to the same muscle group receiving the GnRH immunogen but

at a different site so as to enhance the antibody response with minimized local irritation. For this purpose, a sub-optimal dose of the D17DT conjugate (10 μg) was used. The immune response enhancing supplements containing doses of nMDP were given in amounts of 3 μg and 30 μg, which were dissolved and administered in PBS solution or in an emulsion with Montanide®ISA 703 at a second site from the immunogen. No nMDP was administered to negative control subjects, though a placebo was administered in the form of a PBS solution or a PBS and Montanide®ISA 703 emulsion as the supplement injection. In addition, conventional immunogens were formulated with the conjugate and nMDP combined into single injections in Montanide®ISA 703 emulsions. The nMDP was administered on all three dosing dates.

Test Materials of Example 1

(a) D17DT conjugate (Aphton).

(b) Montanide®ISA 703 (Seppic).

(c) Normuramyl Dipeptide (nMDP) (Peninsula Laboratories).

In accordance with the invention, three separate immunogen formulations were prepared as shown in Table 1. The D17-DT preparation was emulsified with the oily vehicle Montanide®ISA 703 (the immunogenic portion); the adjuvant nMDP was emulsified with Montanide®ISA 703 (the adjuvant supplement portion); and D17-DT in combination with nMDP was emulsified with Montanide®ISA 703 by the conventional method as described below.

Formulations of Example 1

TABLE 1

Immunogen Formulations

Immunogen	Vehicle	D17DT (mg/ml)	D17DT Dose in 0.2ml	nMDP (mg/ml)	nMDP Dose in 0.2ml
1	ISA 703	0.05	10μg	0	0
2	ISA 703	0.05	10μg	0.015	3 μg
3	ISA 703	0.05	10μg	0.15	30 μg

The immunogen emulsions were prepared under clean conditions as a 70:30 (wt:wt) ratio of vehicle to aqueous phase by mixing with the Silverson homogenizer (3 minutes at 8,000 rpm). The resultant emulsions were bottled in sterile multi-dose crimp cap serum vials, with individual vials filled for each dosing date. The materials were stored at 4°C and transported on cold packs.

TABLE 2			
Supplement Formulations			
Supplement Lot	Vehicle	nMDP (mg/ml)	Dose in 0.2 ml
A	PBS	0	0
B	PBS	0.015	3 μg
C	PBS	0.15	30 μg
D	ISA 703	0	0
E	ISA 703	0.015	3 μg
F	ISA 703	0.15	30 μg

Six formulations of supplement were prepared, as shown in Table 2. Two of these (A and D) were placebo controls; four of these (B, C, E and F) contained the adjuvant, nMDP. The supplementary formulations in PBS were prepared by dissolving the nMDP in PBS and filter-sterilizing (0.22 μm filter), then bottled in sterile multi-dose crimp cap serum vials, with individual vials filled for each dosing day. The supplement formulations comprising the Montanide®ISA 703 emulsions were prepared by the same procedures as the immunogenic emulsions, except that immunostimulatory nMDP was substituted for the conjugate in the PBS aqueous phase. The materials were stored at 4°C and transported on cold packs.

According to the these aforementioned protocol, the test immunizations were performed on day 0, 14, and 42 (see Schedule in Table 3 below). Female rabbits were used to perform the novel method of immunization for each of the comparative tests.

TABLE 3					
Rabbit Immunization Schedule					
Rabbit Group	Immunogen	Supplement	Injection 1 Day 0	Injection 2 Day 14	Injection 3 Day 42
1	1	A	0.2ml each	0.2 ml each	0.2 ml each
2	1	B	0.2ml each	0.2 ml each	0.2 ml each
3	1	C	0.2ml each	0.2 ml each	0.2 ml each
4	1	D	0.2ml each	0.2 ml each	0.2 ml each
5	1	E	0.2ml each	0.2 ml each	0.2 ml each
6	1	F	0.2ml each	0.2 ml each	0.2 ml each
7	1	None	0.2 ml	0.2 ml	0.2 ml
8	2	None	0.2 ml	0.2 ml	0.2 ml
9	3	None	0.2 ml	0.2 ml	0.2 ml

The immunizations were performed on 5 rabbits for each subject test group for a total of 45 female rabbits. The bleed schedule provided for a bleeding to be done every 14 days from day 0 through day 68.

The sera were prepared and stored frozen according to standard procedures.

Concerning the dosing procedures, each rabbit in Groups 1 through 6 received two (2) injections on each dosing day, including one (1) injection of Immunogen and one (1) injection of Supplement on the scheduled three (3) dosing days. Thus the combined total number of injections per rabbit of Groups 1 through 6 were three (3) of Immunogen and three (3) of Supplement. Animals in Groups 7 through 9 each received one (1) injection of Immunogen on the scheduled three (3) dosing days, wherein the adjuvant was admixed to the immunogen for a single injectable preparation. The combined total number of injections per rabbit in these groups was three (3) of Immunogen.

The injection doses consisted of 0.2 ml/injection (for each Immunogen, Supplement, and Mix) on each of the three dosing days. After the formulations were allowed to equilibrate to room temperature for 1 hour, each formulation was vigorously shaken prior to dispensing. As indicated above, the dosing schedule provided that the doses 1, 2 and 3 were given on days 0, 14, and 42, respectively. The injection route was intramuscularly (IM) in the hind leg. Thus, the first and second doses were administered to the same leg, and the third dose was administered to the other leg. The nMDP supplement portion was injected as close to the hip (but in the hindleg muscle) as possible, at least 3-4 cm above the site at which the anti-GnRH immunogen portion of the vaccine was injected. Each injection was administered at a muscle site that had not previously been injected. The injection sites were tattooed so as to identify them later.

The Antibody Assay

The sera were tested by an ELISA specifically designed to detect anti-GnRH antibodies. The results of the study of Example 1 showed that the separate injections of immunogen emulsion and the supplement (nMDP adjuvant) emulsion afforded a surprisingly advantageous method for the effective raising of the anti-hormonal antibody titer in the subject animals. For the purpose of clearly determining the improvement effect of this method suboptimal amounts of the antigen were used.

It has been discovered that the immunizing effect of the suboptimal amounts of antigen, i.e., 10 μg of D17DT in Montanide®ISA 703, was much enhanced by the second and third booster injections of the supplement emulsions in the amounts of 3 or 30 μg nMDP in Montanide®ISA 703. For example, after 68 days, that is about 4 weeks after the last (third)

dosing, the anti-GnRH antibody titer rose to about 12,500 when using separate emulsions of 10 µg D17-DT/ISA 703 and 3 µg nMDP/ISA 703, respectively, and to about 26,000 when using similarly 10µg D17-DT/ISA 703 and 30 µg nMDP/ISA 703, respectively. Animals that received the D17-DT antigen and nMDP supplement combined (Immunogen 2 and 3) in a single Montanide®ISA 703 injection responded with antibody titers similar to these groups. Thus, administration of nMDP as a supplement in Montanide®ISA 703 (group 5 and 6) was equally as effective as mixing the nMDP with the conjugate in a single formulation (group 8 and 9). Moreover, antibody titers were about 2 to 4 fold higher than, for example, the titers observed for dosings of an emulsion with 0 µg nMDP indicating the immunopotentiating effect of the supplemental nMDP.

Surprisingly, Groups 1, 2 and 3, which received adjuvant (nMDP) in the form of a PBS solution, did not produce effective levels of anti-GnRH antibody. Thus the nMDP supplement was effective when given as a sustained release emulsion formulation (such as in Montanide®ISA 703) not as an aqueous solution.

The antibody titer assays of the immunized female rabbits are tabulated in Table 4 below.

TABLE 4		Antibody Titers					
Group #	Rabbit #	Day 0	Day 14	Day 28	Day 42	Day 56	Day 68/70
1	1	0	0	132	746	2,878	3,708
	2	0	11	1,388	1,035	2,813	2,757
Immunogen:10µg D17DT in ISA 703	3	0	847	4,211	5,343	12,400	27,600
Supplement:PBS only (Control)	4	0	0	276	1,019	5,940	6,513
	5	0	125	1,090	1,259	2,349	2,118
	Mean	0	34	722	1,015	3,495	3,774
	Median	0	6	683	1,027	2,846	3,233
	S. D.	0	61	613	210	1,647	1,939
2	6	0	0	205	88	957	1310
	7	0	0	567	261	2084	1865
Immunogen:10µg D17DT in ISA 703	8	0	225	432	946	3251	3160
Supplement:3µg nMDP in PBS	9	0	0	360	864	1988	2352
	10	0	402	3,096	2338	4058	3979
	Mean	0	125	932	899	2,468	2,533
	Median	0	0	432	864	2,084	2,352
	S. D.	0	183	1,217	886	1,204	1,056
3	11	0	0	229	547	2186	1962
	12	0	205	1,075	664	1511	1815
Immunogen:10µg D17DT in ISA 703	13	0	0	234	253	984	1709
Supplement: 30 µg nMDP in PBS	14	0	251	348	431	1660	2836
	15	0	55	678	726	1326	4789
	Mean	0	114	472	474	1,585	2,081
	Median	0	103	391	489	1,586	1,889
	S. D.	0	133	406	176	494	514
4	16	0	456	1,741	1014	1828	1343
	17	0	1,524	1,079	1439	3045	1918
Immunogen:10µg D17DT in ISA 703	18	0	319	590	772	940	1407
Supplement: ISA 703 only (Control)	19	0	119	513	654	2703	2681
	20	0	65	623	669	1840	1959
	Mean	0	497	909	910	2,071	1,862
	Median	0	319	623	772	1,840	1,918
	S. D.	0	595	515	329	828	538
5	21	0	152	549	646	1291	1150
	22	0	1,226	2,126	2773	2092	2133
Immunogen:10µg D17DT in ISA 703	23	0	683	2,529	6095	6781	7199
Supplement: 3 µg nMDP in ISA 703	24	0	1,795	13,700	21,600	27,400	30,500
	25	0	2,545	13,300	13,300	21,800	21,500
	Mead	0	1,280	6,441	8,883	11,873	12,496
	Median	0	1,226	2,529	6,095	6,784	7,199
	S. D.	0	935	6,488	8,576	11,971	12,936
6	26	0	1,286	5,777	13,000	21,300	10,300
	27	0	539	8,749	16,100	32,800	20,900
Immunogen:10µg D17DT in ISA 703	28	0	1,673	12,000	15,700	17,900	13,400
Supplement: 30 µg nMDP in ISA 703	29	0	1,384	10,400	10,500	20,700	22,300
	30	0	1,360	6,838	22,300	37,400	21,000
	Mean	3	1,248	8,753	15,520	26,020	17,580
	Median	0	1,360	8,749	15,700	21,300	20,980
	S. D.	6	423	2,539	4,413	8,544	5,373
7	31	0	152	1,796	3885	5998	8827
	32	0	77	819	2559	5915	8874
10 µg D17DT+ 0 nMDP	33	0	948	728	1231	3816	6869
Single Injection in ISA 703	34	0	127	918	981	3358	4241
	35	0	0	481	5913	1395	3549
	Mean	0	255	948	2,914	4,096	6,472
	Median	0	127	819	2,559	3,816	6,869
	S. D.	0	375	501	2,039	1,926	2,500

Group #	Rabbit #	Day 0	Day 14	Day 28	Day 42	Day 56	Day 68/70
8	36	0	1,150	12,000	46,800	28,800	33,100
	37	0	1,014	6,331	12,300	9132	9463
10 μg D17DT + 3μg nMDP	38	0	816	5,025	5886	5406	8625
Single Injection in ISA 703	39	0	157	5,401	7667	8315	8067
	40	0	0	5,720	12,200	10,800	10,700
	Mean	0	497	5,619	9,513	8,413	9,214
	Median	0	487	5,561	9,934	8,724	9,044
	S. D.	0	494	553	3,243	2,256	1,145
9	41	0	1,153	16,500	27,200	50,300	48,100
	42	0	1,765	28,500	27,700	16,300	15,000
10 μg D17DT + 30 μg nMDP	43	0	366	5,496	12,700	16,000	23,800
Single Injection in ISA 703	44	0	360	12,000	19,000	10,100	9,018
	45	0	575	11,800	29,000	17,200	13,100
	Mean	0	844	14,859	23,120	21,980	21,804
	Median	0	575	12,000	27,200	16,300	15,000
	S. D.	0	608	8,573	7,031	16,078	15,660

Gross Pathology Observations

The injection sites were evaluated for gross (macroscopic) appearance of the thigh muscle after injection of test materials. Mean gross pathology scores by group are presented in Table 5. The separate injections of antigen and adjuvant were well tolerated (Groups 1-6), with scores ranging from normal tissue to minimal pathology present. A comparison of injection site reactions on groups paired for dose of adjuvant (Groups 4 vs. 7, 5 vs. 8, 6 vs. 9), indicates that mixing the antigen and adjuvant in a single injection resulted in significantly higher irritation or pathology scores than seen in the corresponding supplement groups. Therefore, separate administration of the nMDP adjuvant led to a marked improvement in injection site tolerance of the immunogen.

TABLE 5

Mean Gross Pathology Scores

Group #	Immunogen Site #1	Supplement Site #1	Immunogen Site #2	Supplement Site #2	Immunogen Site #3	Immunogen Site #3
1	0	0	0	0	0	0
2	0	0.25	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0.25	0	0.75	0.25
5	0	0	0.25	0	0	0.25
6	0.5	0.25	0.25	0.5	1.0	0.5
7	0.25	N/A	0.5	N/A	0.75	N/A
8	0.5	N/A	1.5	N/A	2.0	N/A
9	1.0	N/A	2.5	N/A	3.0	N/A

0—Normal Tissue
1—Minimal Pathology
2—Moderate Pathology
3—Severe Pathology

Intermediate grades are assigned when lesions do not fall unequivocally within the definition of a certain grade.

The above example demonstrates that the present invention provides an improved method of immunization both by increasing the immune response of an immunized animal in terms of producing antibodies, and by reducing undesirable injection site reactions. The method comprises a separate immune response-stimulating composition containing a nontoxic adjuvant, which is administered separately from the actual immunogen. The immunogen itself can be constructed to target the immune response against the effective (i.e., accessible) epitopes of pathogenic organisms as well as other antigens of normal and malignant tissue or cells. One skilled in the art will recognize that the separate steps of immunization of this invention would afford a wide variety of applications and strategies so as to significantly improve the therapeutic success of immunization.

These results are significant, as the capacity to selectively administer supplemental adjuvant affords the physician with additional control over the immunization treatment. This is advantageous, as the principal benefit of nMDP enhancement appear to be expressed in the primary injection (separate study from those described here) when optimal doses are administered; hence, nMDP may not be needed in subsequent injections. Thus, the physician can elect to administer the adjuvant if it is needed to boost the response in a patient with suboptimal responsiveness, as well as to decide upon the optimal dose of adjuvant to administer with the selected dose of vaccine. This would not be an option with the conventional approach, wherein the nMDP is formulated with the conjugate (antigen) at the time of manufacture. Moreover, at lower dosages of conjugate, the formulations are better tolerated, despite being equally immunogenic. Thus, this invention enables the immunization regimen to be tailored to best suit the needs of the individual patient.

Whereas the present examples pertain to the use of water-in-oil emulsions, the skilled formulator would expect that oil-in-water emulsions are also applicable.

In addition to Montanide ISA 703, other metabolizable oily substances such as Montanide ISA 719 as well as Montanide ISA 720 provide stable water-in-oil (70:30 or 50:50) emulsions. An oil-in-water emulsion can be produced by mixing 25 parts of Montanide ISA 25 oily vehicle with 75 parts of aqueous phase.

In addition to the above described examples, the advantageous invention can be envisioned even by one of ordinary skill to encompass effective immunization of animals, in particular, mammals including humans, in defense against various organisms or control of

physiological activities by the many hormones, factors, and receptors involved with the normal and abnormal intercellular regulation.

For example, the advantageous invention can be directed to immunizing against gastrin or the cleaved gastrin peptides (G17 and G34), in accordance with the above-referenced coassigned U.S. Patents No. 5,023,077 and 5,468,494, which are incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. A method for immunization comprising separately administering an immunogenic sustained release composition and an immune response enhancing composition.

2. The method of claim 1, wherein the secondary composition comprises a compound which is effective in stimulating a strong immune response.

3. The method of claim 1, wherein the method elicits a significant anti-immunogen antibody titer increase.

4. The method of claim 1, wherein the immunogenic composition comprises a pharmaceutically acceptable immunogenic emulsion comprising an antigen which comprises an immunomic domain and an immunogenic domain.

5. The method of claim 1, wherein the separate steps of immunizing are administered at an initial dosing and subsequent booster dosings.

6. The method of claim 1, wherein the immunogenic composition comprises a conjugate or complex of an immunomimic domain with an immunogenic carrier.

7. The method of claim 1, wherein the immunogenic composition and the immune response enhancing composition each comprise a pharmaceutically acceptable oily substance.

8. The method of immunization of claim 6, wherein the immunomimic domain comprises an epitope selected from the group consisting of a microbe protein, an autologous eukaryotic cell antigen, an heterologous eukaryotic cell antigen, an enzyme, a cofactor, and a hormone.

9. The method of immunization of claim 7, wherein the oily substance comprises Montanide ISA 703, Montanide ISA 25, Montanide ISA 719, or Montanide ISA 720.

10. The method of claim 1or 2, wherein the immune response enhancing composition comprises an immunostimulating compound.

11. The method of claim 10 wherein the immunostimulating compound is formulated for sustained release.

12. The improved method for immunization of claim 11, wherein the sustained release formulations further comprise liposomes, microparticles, and implantable vehicles for delivery.

13. The method of claim 10, wherein the immunostimulating compound is norMDP, threonyl MDP, LPS, or SLPS.

14. An immunization kit for increasing the immune response to a vaccine target comprising, in separate sustained release preparations,

(i) an immunogenic composition, and

(ii) an immune response stimulating composition.

15. The immunization kit of claim 12, wherein the immunogenic composition comprises an epitope of an immunogen target.

16. The immunization kit of claim 12, wherein the immune response stimulating composition comprises an effective, nontoxic adjuvant.

17. The immunization kit of claim 12, wherein the compositions are kept in separate containers for separate inoculations.

18. The immunization kit of claim 12, wherein the sustained release preparations comprise emulsions, liposomes, microparticles, or implantable vehicles.

19. An improved composition for parental immunization comprising separately (i) a formulation for a sustained release immunogenic composition and (ii) an immune response enhancing composition.

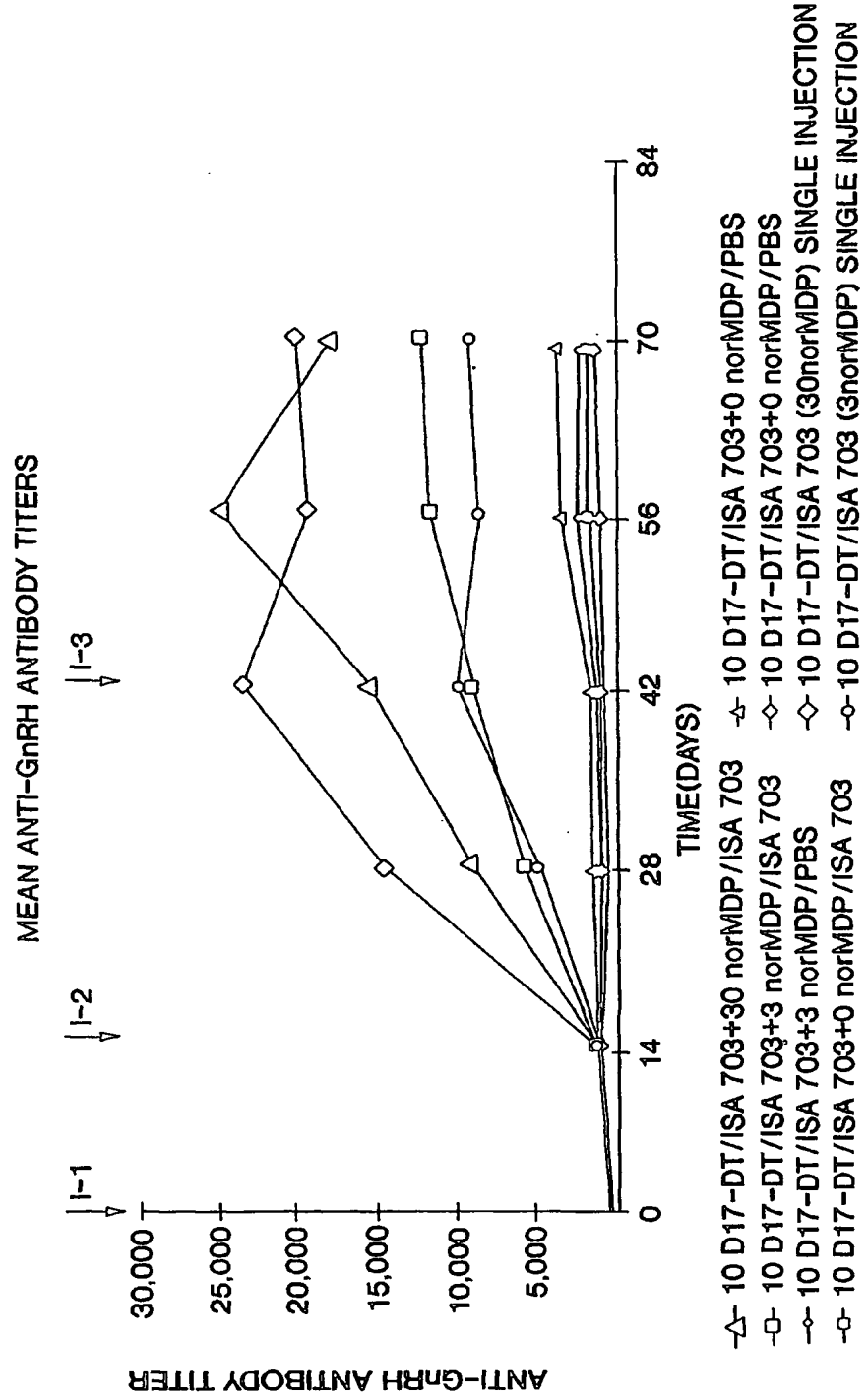
MEAN ANTI-GnRH ANTIBODY TITERS
I-1
I-2
I-3
30,000
25,000
20,000
15,000
10,000
5,000
ANTI-GnRH ANTIBODY TITER
0
14
28
42
56
70
84
TIME(DAYS)
10 D17-DT/ISA 703+30 norMDP/ISA 703
10 D17-DT/ISA 703+3 norMDP/ISA 703
10 D17-DT/ISA 703+3 norMDP/PBS
10 D17-DT/ISA 703+0 norMDP/ISA 703
10 D17-DT/ISA 703+0 norMDP/PBS
10 D17-DT/ISA 703 (30norMDP) SINGLE INJECTION
10 D17-DT/ISA 703 (3norMDP) SINGLE INJECTION

FIG. 1

INTERNATIONAL SEARCH REPORT

Iternational Application No
'CT/US 00/30778

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 July 2001	18/07/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Noë, V

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INTERNATIONAL SEARCH REPORT

ternational Application No
PCT/US 00/30778

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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